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Identification of Inhibitors of PLU-1, a Histone Demethylase that Promotes Breast Cancer Progression

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14. ABSTRACT PLU-1 is a member of the JARID1 family of Jumonji C histone demethylases and specifically demethylates the di- and trimethylated forms of Lys4 in histone H3 (H3K4me2/3). Demethylation of H3K4me2/3 by PLU-1 silences the expression of genes that regulate cell cycle progression in breast cells, promoting invasive cancer. These findings have been corroborated by mouse model studies demonstrating that the activity of PLU-1 is essential to cell proliferation, suggesting that PLU-1 inhibitors hold potential in treating breast cancer. The aim of this project is to express recombinant PLU-1 for use high-throughput screens (HTS) to identify small molecule inhibitors. Toward this goal, multiple constructs encompassing the catalytic domain of PLU-1 have been prepared and tested for soluble expression. The expression tests revealed that the PLU-1 constructs were poorly expressed or insoluble in bacteria. Based on these findings, an alternative approach was adopted wherein we tested the expression of RBP2, an H3K4me2/3-specific demethylase that is highly homologous to PLU-1. These tests demonstrated that most RBP2 constructs were not expressed or insoluble, with the exception of two constructs that showed limited solubility. These findings indicate that additional screening will yield soluble enzymes that can be used in HTS for PLU-1 inhibitors.					
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INTRODUCTION

PLU-1 (also known as JARID1B and KDM5b) has been identified as an oncogene whose expression is upregulated in malignant breast cancers¹. This protein is a member of the Fe(II)- and 2-oxoglutarate (2-OG)-dependent Jumonji C (JmjC) family of histone lysine demethylases and specifically demethylates the di- and trimethylated forms of Lys4 in histone H3 (H3K4me2/3)². H3K4me3 is enriched in the promoter regions and 5' coding regions of actively transcribed genes, whereas H3K4 hypomethylation is associated with silent genes. Consistent with these findings, upregulation of PLU-1 represses the expression of genes that regulate cell cycle progression in breast cells, such as BRCA1, HOXA5, and CAV1^{2, 3}. Correlatively, siRNA-mediated knockdown of PLU-1 has been shown to substantially reduce neoplasia in a mouse mammary tumor model². More recently, PLU-1 has been shown to maintain cells in a proliferative state by silencing genes implicated in cellular differentiation and cell cycle control, including BMI1, EGR1, and p27⁴. Collectively, these findings define a direct relationship between the demethylase activity of PLU-1 and the proliferative capacity of invasive breast tumors, suggesting that PLU-1 inhibitors hold significant therapeutic potential in treating breast cancer.

BODY

PLU-1 is an oncogene that has been implicated in breast and prostate cancer¹⁻³. This protein belongs to the JARID1 family of the JmjC class of histone lysine demethylases, which are Fe(II)-dependent hydroxylases that utilize the co-substrates 2-oxoglutarate and molecular oxygen^{5, 6}. These enzymes catalyze lysine demethylation via a radical-based mechanism that oxidizes the methyl group to produce formaldehyde and the demethylated lysine residue⁶. PLU-1 specifically demethylates H3K4me2 and H3K4me3 to silence genes that regulate cancer cell proliferation²⁻⁴. The aim of this project is to express recombinant forms of PLU-1 for use *in vitro* high-throughput screens (HTS) to identify small molecules that inhibit its demethylase activity. Once PLU-1 is expressed and purified, a fluorescent demethylase assay that is commercially available will be used to screen a library of ~60,000 compounds for inhibitors.

The first phase of this project involves expressing constructs of the PLU-1 catalytic domain for subsequent purification and kinetic characterization. Twelve constructs of mouse PLU-1 were cloned as Mocr fusion proteins for small-scale expression and solubility screening in *E. coli*. The Mocr protein is a newly engineered fusion tag of ~17 kDa in molecular weight that can effectively solubilize recombinant proteins comparable to GST and MBP tags⁷. The Mocr tag is designed as an N-terminal fusion in a pET-based vector and possesses a hexahistidine tag for affinity purification using a Ni or Co sepharose column. A tobacco etch virus (TEV) protease cleavage site has been engineered between the C-terminus of Mocr and the N-terminus of the passenger protein to permit removal of the Mocr tag during purification.



Figure 1: PLU-1 domain structure. PLU-1 is a 1544 amino acid polypeptide that is composed of multiple domains. The N-terminal half of the enzyme (1-751) possesses histone demethylase activity and is composed of five distinct domains: JmjN domain (residues 32-72), ARID domain (97-187), PHD domain (305-359), JmjC domain (453-619), and a C5HC2 Zn finger domain (692-744)². The C-terminal region of Plu-1 contains two additional PHD domains that have been implicated in chromatin binding⁸.

Enzyme	Construct	Mocr Fusion Protein, Molecular Weight (kDa)	Expression Test at pH 6.5	Expression Test at pH 7.5	Expression Test at pH 8.5
PLU-1	30-760	100.9	No Expression	No Expression	No Expression
	30-772	102.3	No Expression	No Expression	No Expression
	30-791	104.5	No Expression	No Expression	No Expression
	77-760	95.5	No Expression	No Expression	No Expression
	77-772	96.8	No Expression	No Expression	No Expression
	77-791	99.1	Insoluble	Insoluble	Insoluble
	302-760	69.4	No Expression	No Expression	No Expression
	302-772	70.7	Insoluble	Insoluble	Insoluble
	302-791	72.9	Insoluble	Insoluble	Insoluble
	362-760	62.8	No Expression	No Expression	No Expression
	362-772	64.1	Insoluble	Insoluble	Insoluble
	362-791	66.4	Insoluble	Insoluble	Insoluble
RBP2	17-744	100.2	Insoluble	Insoluble	Insoluble
	17-756	101.6	No Expression	No Expression	No Expression
	17-775	103.9	No Expression	No Expression	No Expression
	64-744	94.8	Poor solubility	Poor solubility	Poor solubility
	64-756	96.1	Insoluble	Insoluble	Insoluble
	64-775	98.5	Insoluble	Insoluble	Insoluble
	286-744	69.5	Insoluble	Insoluble	Insoluble
	286-756	70.8	No Expression	No Expression	No Expression
	286-775	73.2	No Expression	No Expression	No Expression
	346-744	62.8	Insoluble	Insoluble	Insoluble
	346-756	64.1	Insoluble	Insoluble	Insoluble
	346-775	66.5	Poor solubility	Poor solubility	Poor solubility
SMCX	12-774	104.7	No Expression	No Expression	No Expression
	12-786	106.1	No Expression	No Expression	No Expression
	12-805	108.5	Insoluble	Insoluble	Insoluble
	59-774	99.4	Insoluble	Insoluble	Poor solubility
	59-786	100.7	Insoluble	Insoluble	Poor solubility
	59-805	103.1	Insoluble	Insoluble	Insoluble
	307-774	70.8	Poor solubility	Poor solubility	Poor solubility
	307-786	72.1	No Expression	No Expression	No Expression
	307-805	74.5	No Expression	No Expression	No Expression
	367-774	63.8	Insoluble	Insoluble	Poor solubility
	367-786	65.1	No Expression	No Expression	No Expression
	367-805	67.5	Insoluble	Insoluble	Insoluble
SMCY	12-765	103.4	Insoluble	Insoluble	Poor solubility
	12-777	104.7	Insoluble	Insoluble	Insoluble
	12-796	107.2	Insoluble	Insoluble	Poor solubility
	59-765	98.2	Insoluble	Insoluble	Insoluble
	59-777	99.5	Insoluble	Insoluble	Insoluble
	59-796	101.9	No Expression	No Expression	No Expression
	307-777	70.8	Poor solubility	Poor solubility	Poor solubility
	307-796	73.2	Insoluble	Insoluble	Insoluble
	367-765	62.7	No Expression	No Expression	No Expression
	367-777	64.0	No Expression	No Expression	No Expression
	367-796	66.4	No Expression	No Expression	No Expression

Table 1: Summary of the results of the expression and solubility screens of the Mocr fusion proteins of PLU-1 RBP2, SMCX, and SMCY expressed in Rosetta2 DE3 cells and lysed at different pH values.

Constructs of PLU-1 were designed based on a bioinformatics analysis of its sequence, domain structure, and predicted secondary structure. The N-terminal half of the enzyme contains its JmjC catalytic domain as well as several smaller domains flanking the JmjC domain (Figure 1)². A series of twelve constructs representing the N-terminal half of the enzyme and truncated proteins lacking the JmjN, ARID, and/or PHD domains were designed and cloned as Mocr fusion proteins (Figure 1 and Table 1). The vectors encoding the Mocr-PLU-1 fusions were transformed in Rosetta2 DE3 cells (Novagen) and induced overnight at 20 °C to optimize soluble expression of the recombinant proteins. The cells were harvested the following day and

lysed using a commercial bacterial lysis reagent (Bugbuster, Novagen). Cells were lysed at pH values 6.5, 7.5, and 8.5 to account for the pI values of the Mocr-PLU-1 fusions when assessing protein solubility. The soluble and insoluble fractions of the cell lysate were separated by centrifugation, and the insoluble pellet was solubilized in 6 M urea buffer. The soluble and insoluble fractions were then resolved on an SDS-PAGE gel. A plant Rubisco methyltransferase, which has a molecular weight similar to the Mocr-PLU-1 fusion proteins, was used as a positive control for induction and solubility. A representative solubility test for two Mocr-PLU-1 fusion proteins is depicted in Figure 2.

The solubility tests revealed that seven of the Mocr-PLU-1 fusion proteins were not expressed at an appreciable level (Table 1). The remaining five constructs expressed well but were found in the insoluble fraction (Figure 2), indicating that the proteins formed inclusion bodies when expressed in the bacteria. Due to the poor expression and solubility of the PLU-1 constructs and the need to express large quantities of enzyme for HTS, we examined whether RBP2 (also known as JARID1A and KMT5A), a closely related homolog of PLU-1, could be expressed and purified to serve as a surrogate enzyme in HTS assays. A similar strategy has proven successful in identifying inhibitors of homologous histone methyltransferases using HTS⁹. RBP2 displays identical substrate specificity to PLU-1 in demethylating H3K4me2/3^{10, 11}, and its catalytic domain shares ~58% sequence identity and ~74% sequence conservation with that of PLU-1. Twelve constructs encompassing the catalytic domain of human RBP2 were designed and cloned as Mocr fusion proteins as described above for PLU-1. Solubility tests of the Mocr-RBP2 fusions demonstrated that four of the constructs did not express, six were insoluble, and two showed poor solubility (Table 1). The solubility of these two constructs is insufficient for purification of the enzyme for kinetic characterization and subsequent HTS.

Based on our observation that certain Mocr-RBP2 fusions displayed limited solubility, we cloned and tested the expression and solubility of two additional PLU-1 homologs that are JARID1 H3K4me2/3-specific demethylases: SMCX (JARID1C/KDM5c) and SMCY (JARID1D/KDM5d)¹²⁻¹⁵. SMCX shares ~50% sequence identity and ~65% sequence homology with PLU-1, whereas SMCY has ~51% identity and ~69% homology with PLU-1. Constructs containing the catalytic domains of SMCX and SMCY were cloned as Mocr fusion proteins and tested for expression and solubility as described for PLU-1 and RBP2. Expression and solubility tests of SMCX demonstrated that five constructs did not express, three constructs expressed but were insoluble, and the remaining four constructs displayed limited solubility (Table 1). Similar screening of SMCY revealed that four constructs did not express, four constructs were expressed in insoluble inclusion bodies, and three constructs had marginal solubility.

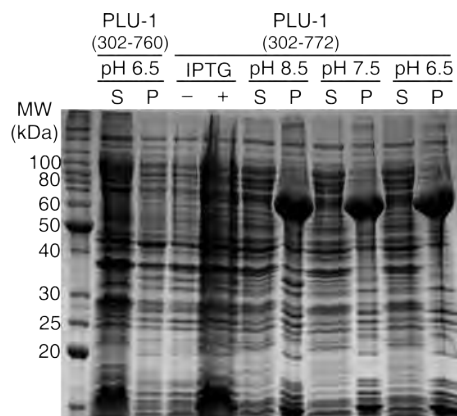


Figure 2: Representative solubility screens for the Mocr-PLU-1 (302-760) and Mocr-PLU-1 (302-772) fusion proteins resolved on a 12% SDS-PAGE gel. Lanes labeled with isopropylthiogalactoside (IPTG - and IPTG +) represent uninduced and induced bacterial samples, respectively. Each Mocr-PLU-1 was tested for solubility at three pH values using buffers containing TRIS pH 8.5, HEPES pH 7.5, or Bis-TRIS pH 6.5. The soluble supernatant (S) and the insoluble pellet (P) are shown for each solubility test.

KEY RESEARCH ACCOMPLISHMENTS

- Expression tests were performed for twelve constructs of the catalytic domain of PLU-1 that were expressed as Mocr-tagged fusions to enhance protein solubility.
- The Mocr-PLU-1 fusion proteins were not expressed at an appreciable level in bacteria or formed insoluble inclusion bodies at three different pH values.
- Additional solubility screens were conducted on twelve constructs of RBP2, a JARID1 H3K4me2/3-specific demethylase that is highly homologous to PLU-1.
- Solubility tests of the Mocr-RBP2 fusions demonstrated that two constructs displayed very limited solubility, whereas the remaining ten constructs did not express or were insoluble when tested under the same conditions as used for PLU-1.
- Expression and solubility tests of the Mocr-SMCX fusions showed that three constructs displayed very limited solubility at pH 8.5, whereas one construct exhibited marginal solubility at the three pH values tested. The remaining constructs did not express to an appreciable level or were insoluble when expressed in *E. coli*.
- Solubility tests of the Mocr-SMCY fusion proteins revealed that two constructs displayed limited solubility at pH 8.5, while one construct exhibited marginal solubility at the three pH values screened. The remaining constructs either did not express or were insoluble.

REPORTABLE OUTCOMES

The data described in this report will be presented at the 2011 Era of Hope Conference in Orlando, Florida.

PERSONNEL RECEIVING SUPPORT

Dr. Raymond C. Trievel (PI), Associate Professor of Biological Chemistry

Dr. Paul Del Rizzo, Postdoctoral Fellow, Department of Biological Chemistry

CONCLUSION

In the first phase of our project to screen for inhibitors of PLU-1, we tested the twelve constructs of the PLU-1 catalytic domain that had been cloned with a Mocr fusion tag for expression and purification from bacteria. The recombinant Mocr-PLU-1 fusion proteins did not express at a detectable level or were insoluble despite tests at different pH values. Due to the requirement to express large quantities of PLU-1 enzyme for HTS, an alternative approach was chosen in which we tested the expression of constructs of RBP2, a JARID1 demethylase that shares high sequence homology with PLU-1. Expression and solubility tests of Mocr-RBP2 fusion proteins demonstrated that two constructs displayed very limited solubility, whereas the remaining ten constructs were either insoluble or did not express. Based on these results, constructs of the PLU-1 homologs, SMCX and SMCY, were cloned and tested for expression and solubility. Four Mocr-SMCX and three Mocr-SMCY constructs showed limited solubility in *E. coli* Rosetta2 DE3 cells. Additional optimization of the expression conditions of the marginally soluble RBP2, SMCX, and SMCY constructs may yield sufficient enzyme for preliminary kinetic characterization and small-scale HTS. Alternatively, the expression of PLU-1 or its homologs in a eukaryotic cell line will need to be explored to obtain a sufficient quantity of enzyme to undertake large-scale HTS for small molecule inhibitors.

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Appendices

- A. Del Rizzo P and Trievel RC. Characterization of Histone Demethylases Involved in Breast Cancer Progression, 2011 Era of Hope Conference, Orlando, FL.

APPENDIX

2011 Era of Hope Conference

Characterization of Histone Demethylases Involved in Breast Cancer Progression

Paul Del Rizzo and Raymond C. Trievel, Department of Biological Chemistry, University of Michigan Medical School

PLU-1 had been identified as an oncogene with elevated expression in malignant breast cancers. Also known as JARID1B and KDM5b, this enzyme is a member of the Fe(II)- and 2-oxoglutarate (2-OG)-dependent Jumonji C (JmjC) family of histone lysine demethylases (HDMs) and has been shown to specifically demethylase di- and tri-methyl lysine 4 from histone H3 (H3K4me2/3). Methylation at the H3K4 position is indicative of transcriptionally active chromatin, whereas removal of the methylation from this site is associated with silent genes. It has been shown that upregulation of PLU-1 results in the repression of genes that control breast cancer cell cycle progression, such as BRCA1, HOXA5, and CAV1. In addition, knockdown of PLU-1 has been shown to reduce neoplasia in a mouse mammary tumor model. PLU-1 has also been shown to maintain cells in a proliferative state by silencing genes implicated in cellular differentiation and cell cycle control, including BMI1, EGR1, and p27. Thus, there exists a direct relationship between the PLU-1 demethylase activity and cell proliferation in breast tumors, suggesting that the identification of PLU-1 inhibitors could hold significant therapeutic potential in the treatment of breast cancer. For this reason, we propose to use high-throughput screening (HTS) to identify potential inhibitors of this enzyme.

A number of recombinant clones of human PLU-1 were generated to identify a stable, soluble form of the protein that contains the catalytic domain of PLU-1, which is suitable for purification, kinetic analysis, and HTS for small molecule inhibitors. These clones were generated as fusion proteins to facilitate their expression and stability in *Escherichia coli*, as well as to assist in purification of the constructs. The clones were transformed and expressed in *E. coli* cells, which were then lysed and their whole-cell contents analyzed for presence of the over-expressed soluble protein. The relative amounts of the construct of interest in the soluble and insoluble fractions were analyzed at pH 6.5, 7.5, and 8.5, as visualized by SDS-PAGE. Proteins found to be sufficiently soluble would be expressed, purified, and subsequently screened for inhibitors from a library of over 60,000 chemicals using a commercially available fluorescent demethylase assay.

Twelve constructs containing the catalytic domain of PLU-1 were cloned as fusion proteins and tested for expression and solubility in bacteria. The solubility screens have shown that a number of the PLU-1 constructs failed to express at an appreciable level, whereas some expressed, but remained insoluble upon cell lysis. Therefore, in a similar manner, fusion proteins of the catalytic domains of PLU-1 homologs were cloned and screened for solubility. This resulted in the identification of proteins that have limited solubility, and may prove to be suitable for purification, kinetic analysis, and inhibition HTS for small molecule inhibitors. It is our expectation that identification of inhibitors for PLU-1 or related homologs will serve as a starting point for the development of a novel class of drugs designed for the treatment of breast cancer.